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14. ABSTRACT
This report is on the second year of our 2011 Tuberous Sclerosis Complex Research Program Idea Development Award (07/12-6/15). The original goals are to explore the potential mechanism for epileptogenesis in Tuberous Sclerosis Complex (TSC) disease, with a focus on altered astrocyte-neuronal interactions caused by astrocyte-specific TSC deficiency. Our hypothesis is that abnormal cells in non-tuber cortex might form an abnormally excitable network that underlies seizure generation in TSC. Epileptogenesis in non-tuber neural tissue in TS may thus arise by an imbalance of decreased inhibitory and increased excitatory synaptic transmission. Astrocytes could also regulate neuronal excitability by glutamate uptake and other means that alter expression and function of synaptic receptors for glutamate, or by altering the number of synapses. A deficiency of GABAergic interneurons, a possible downstream consequence of altered mTOR activity in astrocytes, may further contribute to the early onset and severity of seizures in TS.

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Progress report

GRANT10931149 PI: David Sulzer; Co-PI: James Goldman

Altered Astrocyte-Neuron Interactions and Epileptogenesis in Tuberous Sclerosis Complex Disorder

1. INTRODUCTION

This report is on the third year of our 2011 Tuberous Sclerosis Complex Research Program Idea Development Award (07/12-6/15). The goals are to explore potential mechanisms underlying epileptogenesis in Tuberous Sclerosis Complex (TSC) disease, with a focus on altered astrocyte-neuronal interactions caused by astrocyte specific TSC deficiency. Our hypothesis is that abnormal cells in non-tuber cortex might form an abnormally excitable network that underlies seizure generation in TSC. Epileptogenesis in non-tuber neural tissue in TS may thus arise by an imbalance of decreased inhibitory and increased excitatory synaptic transmission. Astrocytes have been reported to regulate neuronal excitability by glutamate uptake and other means that alter expression and function of synaptic receptors for glutamate, or by altering the number of synapses.

To address a specific role for astrocytes in regulating synaptic function during development, we chose to use a mouse GFAP (mGFAP) promoter sequence directing expression of a Cre-recombinase in most astrocytes and a subpopulation of the adult stem cells in the subventricular zone. Previous studies have reported that there is no targeting of postnatal or adult neural stem cells or their progeny in the hippocampus or other brain regions, rendering these mice particularly useful for selective targeting of astrocytes. We unexpectedly found, however, that there are indeed recombinant neurons that are derived from GFAPcre expressing progenitors and are deficient for TSC1. This provides an important new aspect of analysis: in our new neuroglial TSC1 deficient mouse model, we are able to compare directly and in the same brain region the effect of neuronal-intrinsic mTOR activation of synaptic activities on wild-type and recombinant neurons, as well as the effects of Tsc1-deficient astrocytes on neuronal morphology and neuronal activity associated with seizures.

2. KEY WORDS

epilepsy, seizure, tuberous sclerosis, autophagy, mTOR, astrocytes, dendrites

3. ACCOMPLISHMENTS

Major goals:

Our initial findings in the mouse GFAP-cre-mediated Tsc1 conditional knockout ($TSC1^{mGFAPCre}$ CKO) mice include: 1)$TSC1^{mGFAPCre}$CKO mice develop spontaneous clinical seizures at the age of 2.5 months; b) $TSC1^{mGFAPCre}$CKO mice show astrogliosis, activated mTOR signaling in astrocytes and recombinant neurons, and enlarged cellular size of astrocytes and a few enlarged neurons; C) Glutamate transport and potassium buffering functions are intact in $TSC1^{mGFAPCre}$CKO astrocytes in young mice before the occurrence of behavioral seizures; D) $TSC1^{mGFAPCre}$CKO mice exhibit abnormal excitatory synaptic transmission; E) they display synaptic damage induced by spontaneous seizures; and F) increased spine density on recombinant pyramidal neuron dendrites prior to the onset of spontaneous seizures.

What was accomplished:

We then fully characterized neuronal and astrocyte phenotypes in our $TSC1^{mGFAPCre}$CKO mice, at a range of ages before and after the occurrence of clinical seizures. Using a dTomato-expressing cre reporter Ai9 mouse line, we identified dTomato-positive, recombinant pyramidal neurons in the superficial isocortical layers and in the hippocampal pyramidal layer, indicating a radial glial origin of these late-born excitatory neurons. In $TSC1^{mGFAPCre}$CKO mice, most of these recombinant neurons expressed high levels of pS6 at the age of 1 month, suggesting an TOR hyperactivity in response to the loss of TSC1 alleles. With age, some recombinant neurons maintained high levels of pS6 and displayed increased soma volume and increased dendritic trees. Interestingly, some recombinant neurons displayed a reduction in pS6 levels, which is atypical for mTOR

hyperactivity, and displayed a normal soma size and morphology, suggesting that these neurons possess a negative feedback mechanism that in part counteracts the effect of the TSC1 deletion.

Our data do not support a contribution of astrocyte glutamate uptake and potassium buffering dysfunction in epileptogenesis in our neuroglial TSC-deficient mouse models: glutamate and potassium uptake remains intact in TSC1 deficient astrocytes in our TSC1^{mGFAPCre}CKO mice prior to the onset of clinical seizures. Astrocytes do show increased GFAP and S100b immunoreactivities by 1 month of the age, and this increase becomes more pronounced with age. However, astrocytes do not show decreases in glutamate transporter currents, and GLT-1 and GLAST immunoreactivities until 3.5 months of the age (as we reported from the first year of the award). A slight decrease in astrocyte uptake of synaptically evoked glutamate appears after the occurrence of clinical seizures; these data suggest that the astrocyte pathology evolves slowly in our mouse model. It is possible that the major changes in astrocytes do not manifest until during or after the onset of seizures, suggesting that the seizures produce the major pathology in astrocytes. Nevertheless, the consequent astrocyte pathology may itself contribute to the ongoing seizure activity.

To approach consequences of this mutation on synaptic neurotransmission, we then conducted electrophysiological recordings of miniature EPSCs, evoked EPSCs and paired pulse facilitation in wt and TSC1CKO mice at CA3-CA1 synapses at the age of 3.5 months. We found in the mutants: 1) an increased frequency and amplitude of mEPSCs / evoked EPSCs in CA1 pyramidal neurons, indicative of increased neuronal excitability; 2) decreased paired pulse facilitation (PPF) in CA1 pyramidal neurons, suggesting an enhanced probability of presynaptic neurotransmitter release; 3) by recording high frequency stimulation evoked EPSCs, we found an increased amplitude and increased decay time: this value indicates the amount of glutamate released at the synapse, the amount of extracellular glutamate molecules available for transport, and the expression of transporter proteins. Together, these independent findings confirm an increase in excitatory neural transmission in our TSC1^{mGFAPCre}CKO mice during epileptogenesis.

We found that mGFAPCre expressing recombinant dentate granule cells were of normal size at all times, and there is no detectable mossy fiber sprouting in the knockout mice, which has been reported to be correlated with epilepsy. These data suggest that granule cells may rely on non-mTOR mechanisms to regulate cell size. Alternatively, because of turnover of these cells, it is possible that they do not have enough time to increase over their limited lifespan.

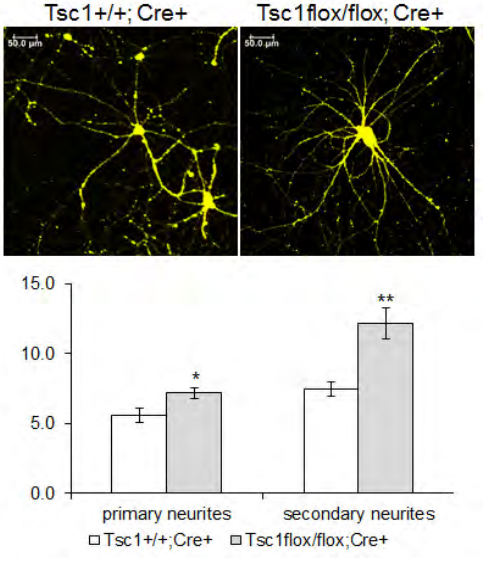


Figure 1. Increased dendritic tree complexity in cultured Tsc1CKO neurons.

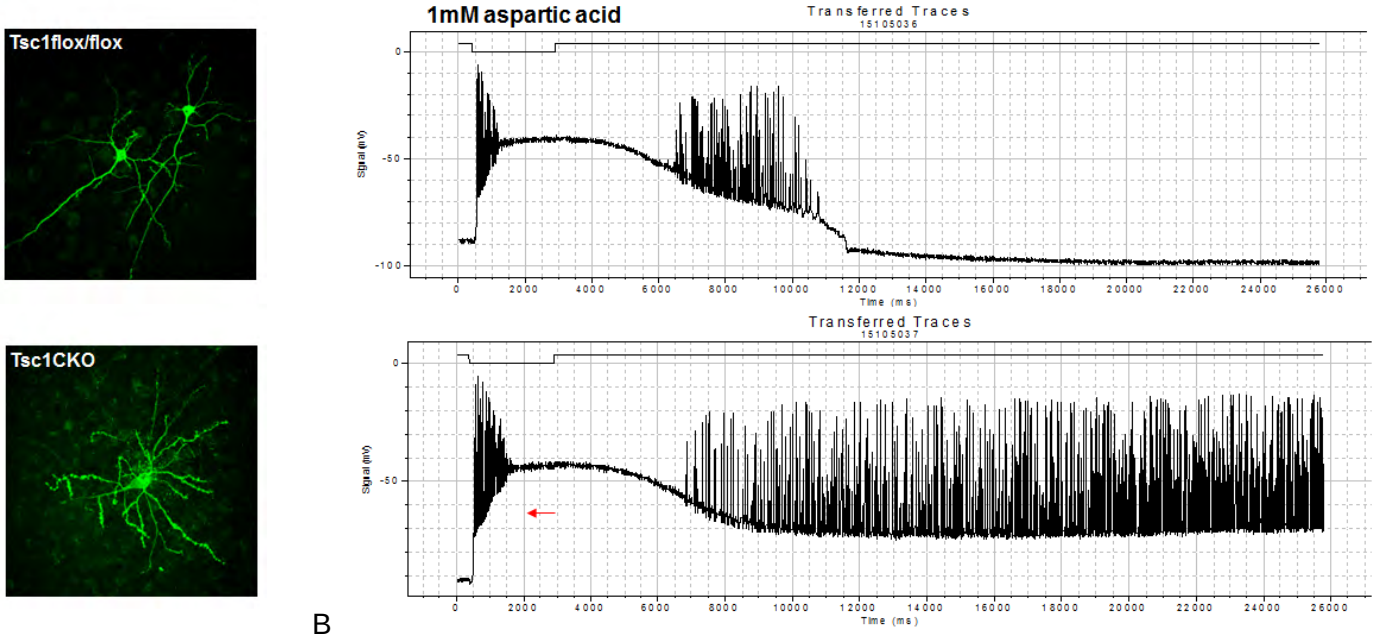
We also tested whether a failure in pruning excessive excitatory synapses may contribute to the pathogenesis of epilepsy in TS by producing neuronal over-excitability. We examined excitatory synaptic density by DiOlistic labeling and immunohistochemistry at age of 1 month, before the occurrence of clinical seizures. Our data showed increased spine density and increased pre and post-synaptic protein puncta in TSC1^{mGFAPCre}CKO CA1 sector.

An alternative mechanism that may explain how epilepsy develops in our TSC1^{mGFAPCre}CKO mice is due to the enhanced intrinsic neuronal hyperexcitability, especially in those recombinant neurons deficient for TSC1 and expressing high level of pS6. Recently, we have concentrated on the characterization of anatomic and physiological properties of mGFAPcre recombinant (dTomato+) and non-recombinant (dTomato-) neurons in the TSC1^{mGFAPCre}CKO; dtomato+ mice, using DiOlistic labeling and patch clamping recording techniques. We have confirmed in TSC1^{mGFAPCre}CKO mice that TSC1-deficient neurons exhibit more dendritic spines than wild type, non-recombinant neurons; yet, the latter show a similar spine density to pyramidal neurons in TSC1 wt, mGFAPCre expressing Ai9 dTomato mice. These data suggest that the increased excitability in TSC1^{mGFAPCre}CKO mice might be due to Cre-expressing recombinant excitatory neurons.

Our recent study (Tang et al., Neuron 2014) in CamkII-cre-mediated, postnatal Tsc1-deficient neurons showed an increased dendritic spine density, with no significant changes in dendritic tree complexity. Thus, for this

study, we examined whether Tsc1 deficiency before the neurite biogenesis would impact the complexity of the dendritic tree. We crossed the Tsc1flox/flox mice to Ai9 cre reporter mice (Jackson Lab: Jax #007909) to obtain Tsc1flox/flox;Ai9+ pups and Tsc1+/+;Ai9+ pups. Ai9 mice carry a *loxP*-flanked STOP cassette that prevents transcription of the downstream red fluorescent protein variant (tdTomato). We transfect primary neurons from Tsc1flox/flox:Ai9+ mice or Tsc1+/+;Ai9+ mice with Cre- plasmids before plating. Cre recombinase will drive dTomato expression**,** which allows imaging dendritic spines in mature neurons in vitro, and removes the Tsc1 gene from Tsc1flox/flox neurons. Compared to Tsc1+/+; Cre-expressing control neurons, Tsc1flox/flox;Cre-expressing Tsc1 CKO neurons exhibit significantly more primary and secondary dendrites, causing them to appear “dysplastic”.

The increased dendritic complexity in TSC1CKO recombinant dysplastic neurons was confirmed in brain slices using luciferase yellow injection (**Figure 2A**). Local pressure injection of a weak NMDA receptoragonist aspartic acid (1mM, 4sec, 10Kpa) was used to induce epilepticform activities in acute TSC1mGFAPCre CKO brain slices. Aspartic acid induced after-discharges in both Tsc1flox/flox non-recombinant neurons and Tsc1CKO recombinant dysplastic neurons. However, dysplastic neurons show prolonged slow-depolarization after initial burst-firing induced by aspartic acid, which was followed by a sustained after-discharge (**Figure 2B**). These data suggest that TSC1CKO dysplastic neurons exhibit long lasting seizure-like activity.



A B

Figure 2. Prolonged discharge observed in the cortical pyramidal neurons in Tsc1mGFAPCreCKO mice. (A) Represented luciferase yellow injected normal size wild type (upper) and dysplastic Tsc1GFAPCreCKO (lower) neurons pyramidal neurons in cortical layers II-II. (B) Induction of electrographic seizures in dTomato negative (Upper) and positive (Lower) neurons in Tsc1GFAPCreCKO mice.

We then examined Tsc deficiency in excitatory projection neurons using a CamKII-Cre mediated Tsc1CKO mouse line. By patch clamp recording from cortical layer II-III pyramidal neurons, we found that there was an increased after depolarization likely to be due to apamin-sensitive SK channels (**Fig 3A**). Consistently, this leads to greater activity with a higher frequency of action potentials (**Fig 3B**), which suggests increased propensity for epileptiform activity. Thus, Tsc deletion directly in neurons has effects that parallel the deletion induced by the mGFAP-cre.

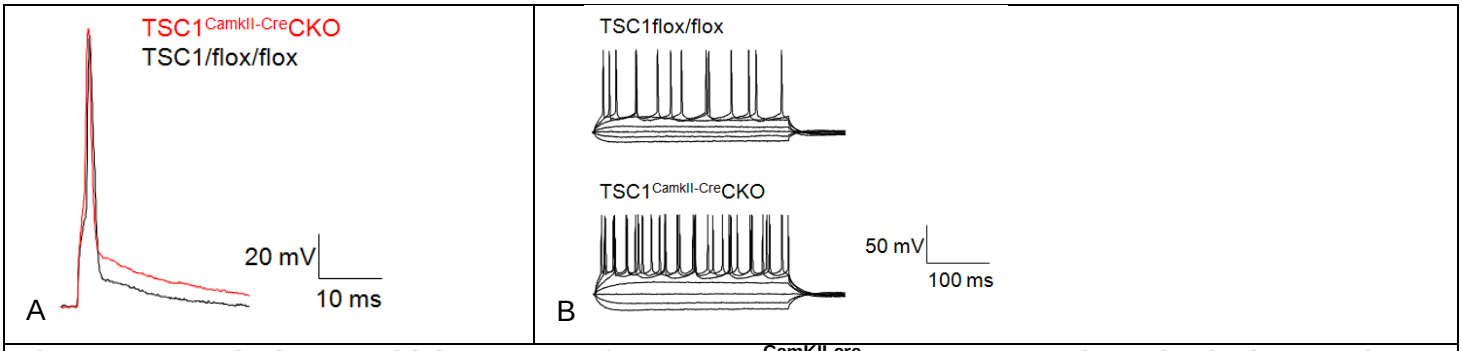


Figure 3. Cortical pyramidal neurons from TSC1$^{CamKII\text{-}cre}$CKO mouse show intrinsic membrane hyperexcitability. A. An enhanced afterdepolarization (ADP) after a single action potential (AP) induced with a short depolarizing current pulse (2 ms, 2 nA) was observed only in pyramidal neurons from TSC1$^{CamKII\text{-}cre}$CKO mouse, but not in pyramidal neuron from TSC fl/fl mouse. B. Example of voltage response to current injection through patch electrode (-200pA to 400 pA , 300ms). The number of action potential was greater in TSC1$^{CamKII\text{-}cre}$CKO pyramidal neurons than in control neurons in response to 200, 300 and 400pA current injection.

The results above indicate changes in intrinsic properties due to TSC deletion, but not changes in response to excitatory input that would drive cortical neuron activity in genuine physiological conditions. Thus, we activated NMDA receptors to determine changes in response. There was as expected a far higher frequency of action potentials in the mutant line, with a highly prolonged membrane depolarization (**Fig 4**). This confirms that there is a substantially higher response to synaptic input in mutant neurons.

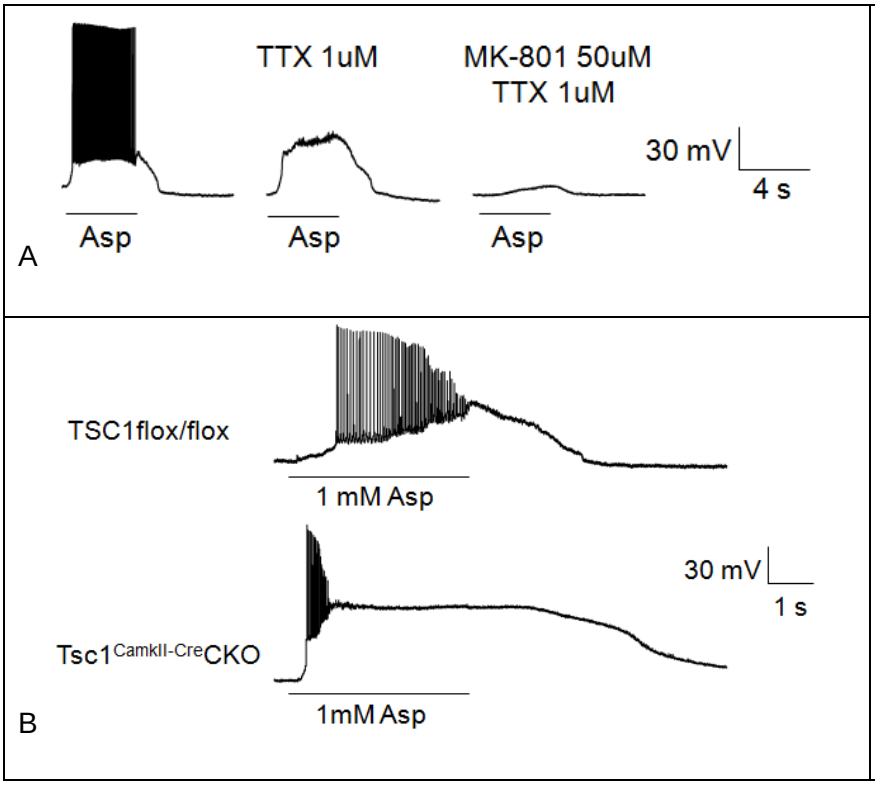


Figure 4. The NMDA receptor agonist aspartic acid (1 mM) induces a prolonged membrane depolarization in cortical pyramidal neuron from Tsc1$^{CamkII\text{-}Cre}$CKO mouse. A. Focally applying aspartic acid evoked action potential firing and membrane depolarization in cortical pyramidal neurons (PYN). TTX (1uM) blocked the induced action potential firing, but not membrane depolarization. The NMDA receptor antagonist (MK-801) almost completely blocked membrane depolarization. B. Representative trace shows the membrane response to aspartic acid in cortical pyramidal neurons fromTSC1/fl/fl mouse (upper panel). Membrane depolarization induced by aspartic acid (1mM) was significantly prolonged in cortical pyramidal neuron (PYN) from Tsc1$^{CamkII\text{-}Cre}$CKO mouse (low panel).

Opportunities for training and professional developement : Nothing to Report.

Dissemination to communities of interest: Nothing to Report

Work for next period:

We have asked for and received a one year no cost extension for this project and will use this period to complete data analysis, some EEG recordings, and submit the papers.

4. IMPACT

We have asked for and received a one year no cost extension for this project and will use this period to complete data analysis, some EEG recordings, and submit the papers. To date, the major findings from the project are:

1) $TSC1^{mGFAPcre}$ CKO mice develop spontaneous seizures at the age of 2-3 months of age.
2) *Tsc1* gene depletion results in astrocytic mTOR hyperactivation and reactive astrogliosis;
3) Postnatal deletion of the *Tsc1* gene does not interfere with the overall capacity of astrocyte glutamate uptake and potassium buffering. However, synaptically-invoked astrocyte glutamate uptake may be impaired due to the lack of TSC1 in astrocytes.
4) Astroglial *TSC1* deletion leads to increased basal and evoked excitatory synaptic transmission during epileptogenesis;
5) Astroglial *TSC1* deletion causes an increase in excitatory synapses prior to the occurrence of clinical seizures.
6) These responses can be replicated if *TSC* is deleted directly from pyramidal neurons, leading to both greater intrinsic excitability and much higher response to excitatory input. Together, these are likely to lead to the initiation and prolongation of epileptiform actitivity.

5. CHANGES/PROBLEMS

Nothing to Report

6. PRODUCTS

Publications, conference papers, and presentations

1. Tang G, Gudsnuk K, Kuo SH, Cotrina ML, Rosoklija G, Sosunov A, Sonders MS, Kanter E, Castagna C, Yamamoto A, Yue Z, Arancio O, Peterson BS, Champagne F, Dwork AJ, Goldman J, Sulzer D. (2014). Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. Neuron. 2014 Sep 3;83(5):1131-43.. Epub 2014 Aug 21

2. **Tang G,** et al. A mouse model of tuberous sclerosis with spontaneous seizures. The 43rd Annual Neuroscience meeting in San Diego, USA. (2013)

N.B. Other papers are under preparation from this project, which has a one year no cost extension.

our website is:

http://sulzerlab.org

There are no new technologies, patents, inventions or licenses.

There are no products. Note however that the above research provides a basis by which TSC mutation causes epilepsy and indicates that substitution for TSC function may provide therapy.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*David Sulzer*
Project Role:	*PI*
Nearest person month worked:	*1*
Contribution to Project:	*Oversaw project*

Name:	*James Goldman*
Project Role:	*co-PI*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Goldman participated in all planning and data analysis.*

Name:	*Ahrom Ham*
Project Role:	*Postdoctoral scientist*
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Ham conducted the immunolabel and Western experiments*

Name:	*Dana Mrejeru*
Project Role:	*Postdoctoral scientist*
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Mrejeru performed electrophysiology experiments*